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# Long-Term *Ex Situ* Conservation of Biological Resources and the Role of Biological Resource Centers

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## Summary

The establishment and maintenance of biological resource centers (BRCs) requires careful attention to implementation of reliable preservation technologies and appropriate quality control to ensure that recovered cultures and other biological materials perform in the same way as the originally isolated culture or material. There are many types of BRC that vary both in the kinds of material they hold and in the purposes for which the materials are provided. All BRCs are expected to provide materials and information of an appropriate quality for their application and work to standards relevant to those applications. There are important industrial, biomedical, and conservation issues that can only be addressed through effective and efficient operation of BRCs in the long term. This requires a high degree of expertise in the maintenance and management of collections of biological materials at ultra-low temperatures, or as freeze-dried material, to secure their long-term integrity and relevance for future research, development, and conservation.

**Key Words:** Biological resource centers; preservation; microorganisms; cell lines; tissues.

## 1. Introduction

Collecting examples of different types of organisms has been the pursuit of scientists and amateur collectors for centuries. This activity was originally stimulated by man's curiosity regarding the natural diversity of "his" environment, but for well over a century scientists have been collecting strains of animals, plants, and microorganisms with specific scientific and technical aims relating to taxonomy, infectious disease, and biochemistry. The first collection of microorganisms for industrial use was established by Kral in 1869 and collections of plants and other organisms developed based on the maintenance of examples of each strain or species under controlled laboratory or field conditions.

From: *Methods in Molecular Biology, vol. 368: Cryopreservation and Freeze-Drying Protocols, Second Edition*
Edited by: J. G. Day and G. N. Stacey © Humana Press Inc., Totowa, NJ

However, such collections of actively growing cultures suffered from complications, such as adaptation of the organism to the in vitro environment, genetic mutations, contamination, and accidental loss of cultures. Clearly a mechanism for arresting growth to reduce such risks was needed.

The 1800s also saw tremendous expansion in our scientific knowledge and engineering capabilities with the consequent development of new techniques, including the compression of gases to the liquid state, which enabled the field of cryobiology to develop rapidly. The ability to use ultra-low temperatures to prevent degradation of biological materials has probably been utilized by man for millennia, and further scientific observations from the 17th and 18th centuries paved the way for discoveries in cyropreservation technology in the 20th century. These led to successful and reliable methods for the preservation of both prokaryotic and eukaryotic organisms so they could be stored indefinitely in a viable and unchanging state of "suspended animation." The preservation of bacteria and fungi had been established by pioneers from the 19th and early 20th centuries, subsequent work of people like Polge et al. ***(1)*** for preservation of animal cells, and Sakai ***(2)*** for plant cells can be viewed as key milestones in the development of cryopreservation processes. This pioneering work has been improved and refined with new approaches and fundamental research into cryobiology that has enabled the preservation of diverse and complex cell and tissue cultures as exemplified in the protocols provided in this book.

Today, culture collections, or more broadly, biological resource centers (BRCs), are a mixture of academic, public service, private, government and commercial activities that deliver important characterized cultures as "seed" stocks:

1. For the development of industrial processes.
2. As reference strains for biological assays and published scientific literature.
3. As type strains for taxonomical studies.
4. As centers for conservation of biodiversity.

In this chapter, we shall outline some of the important principles and challenges involved in the establishment and long-term maintenance of collections of cryopreserved biological materials and cultures.

## 2. Fundamental Principles for BRCs

There are three fundamental features of collections of biological materials that must be sustained to establish the value of stored material: (1) purity (freedom from contaminant organisms); (2) authenticity (correct identity of each strain), and (3) stability, including correct functional characteristics. Purity of a strain is critical to avoid erroneous data. However, in some situations purity

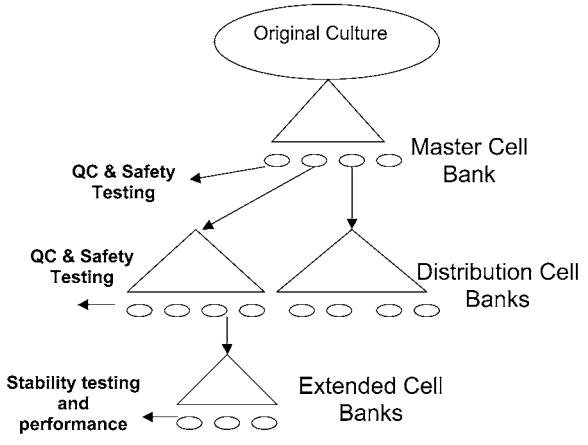


Fig. 1. Scheme of the "master" and "working" (or "distribution") bank system. Each "bank" comprises a number of containers (⬭) each containing a sample of cryopreserved cells from a homogenously mixed single batch of harvested cells. Extended cell banks can also be established to enable testing of culture characteristics at different passages in parallel experiments. This banking scheme can be used to ensure that cultures are subjected to minimum passages in vitro before distribution to researchers. It can also act as the basis for a process map that can be used to establish a quality assurance system with appropriate traceability for bank preparation and quality control ***(9)***.

can be very difficult to establish as the organism may have a saprophytic relationship with other organisms and cannot survive as a pure culture (e.g., certain protists, saprophytic fungi), or can only be preserved within other cells, e.g., malaria ***(3)***. Authenticity is usually based on certain stable phenotypic or genotypic characteristics and will vary for each group of organisms. However, some DNA profiling (e.g., multilocus DNA fingerprinting, short tandem repeat analysis ***[4]***, or gene sequencing techniques (e.g., cytochrome *c* oxidase sequencing ***[5]***, "ribotyping" ***[6]***) provide some generic approaches that can be applied across a broad range of organisms in some cases. For cultures maintained in vitro, failure to maintain their phenotypic and genotypic features on serial passage is a serious concern as cultures (notably viruses, pathogenic bacteria, and cell lines ***[7]***) are known to undergo irreversible changes if maintained in culture for long periods.

Accordingly, it is important that BRCs maintain practices to ensure that the samples of organisms that they hold and distribute sustain these important characteristics. This can be achieved through the adoption of working practices including establishment of master and distribution banks for each organism (**Fig. 1**) ***(8)***, and a robust quality control system to provide assurance that each

bank prepared meets the criteria outlined. The specific quality control regime will vary depending on the particular characteristics of the type of culture. A risk assessment will need to be performed relating to the history of each culture and the more general scientific experience with that culture type. Examples of such considerations is given in **Table 1**.

These requirements have been enshrined in best practice guidelines for culture collections such as the World Federation for Culture Collections' Standards ***(14)***. These identify the minimum standards to ensure an appropriate level of quality of materials available from BRCs that generally seek to exceed them. In addition, there may be specialist guidance for the maintenance of the certain types of cell culture and there are also legally binding regulations and ethical issues for some BRCs as outlined in **Table 2**.

## 3. Important Long-Term Roles for BRCs

The provision of cultures from BRCs is of little value unless these cultures are accompanied by information on their identity, provenance, and characteristics. Culture collections are important store houses of information on cultures that can be accessed by direct contact with individual collections or contacting microorganism database organizations such as MIRCEN at http://wdcm.nig.ac.jp/. This information resource can grow with time as work on cultures from BRCs is published. BRCs also engage in research and development on the technologies involved in the banking of cultures, and are very often the only locations where this type of specialist research and development is maintained. Accordingly, BRCs are often valuable repositories for up-to-date information topics such as biosafety, shipment, characterization, preservation, and taxonomy (*see* www.wfcc.info). They are also frequently the drivers for training and education in such areas.

Many BRCs provide vital services for *ex situ* conservation of biodiversity through the preservation, storage, and documentation of endangered plant tissues (Chapter 12), seeds (Chapter 13), and animal gametes and embryos (Chapters 21–23). Such resource centers could potentially regenerate species that are lost to their natural habitat. The field of biotechnology and biomedicine is also reliant on long-term storage of viable cells to secure reliable seed stocks for the manufacture and testing of products. Furthermore, such long-term storage is critical to secure intellectual property by supporting patent applications where the preserved cells must remain viable for at least 30 yr ***(16,21)***.

## 4. Assuring the Success of Long-Term Storage

### *4.1. Experience and Critical Issues*

It is often assumed that once an organism has been successfully preserved in a viable state in liquid nitrogen that it will remain viable indefinitely at ultra-low

**Table 1**
**Standards That May Apply to Biological Resource Centers**

| Characteristic | Typical methods for bacterial strains | Typical methods for plant germplasm | Typical methods for animal cell lines |
|---|---|---|---|
| Purity | Broth and agar culture to reveal pure growth | Broth and agar culture to reveal absence of bacteria and fungi | Broth and agar culture to show no growth of bacteria and fungi ***(12)*** Tests for mycoplasma ***(12)*** and viral contamination (e.g., electron microscopy, molecular detection, inoculation of animals or test cell lines) ***(13)*** |
| Authenticity | Gram stain reaction, colony morphology, biochemical genotyping, biochemical reactions, ribosomal gene sequence | Morphology of cells/structures, genotype, expression profile of "secondary products," DNA genotype ***(10)*** | DNA fingerprint, karyology, isoenzyme analysis, cytochrome *c* oxidase sequence ***(4)*** |
| Stability/functional capacity (cultures recovered from cell banks reproducibly show key functions) | Antibiotic susceptibility, absence of mutations by gene sequencing | Retain ability to regenerate plants, sustained expression of "secondary products," absence of "sports" ***(10,11)*** | Antibody secretion by hybridomas, susceptibility to viruses, karyology |

**Table 2**
**Standards for Cell Banking**

| Type of culture/application | Standards that may be applicable |
|---|---|
| Research and development | National regulations for handling, containment, and disposal of pathogenic strains<br>National regulations on genetically modified organisms<br>Good cell culture practice ***(15)*** |
| Organisms deposited in support of patent applications | Budapest treaty ***(16)*** |
| Plant cells and tissues | National and international regulations on import and export of pathogens (http://www.wfcc.info) and endangered species (http://www.cites.org/) |
| Animal cells and tissues | National and regulations on appropriate records of licenses, procedures, and staff training<br>National and international regulations on import and export of pathogens and endangered species (*see* above) |
| Human cells and tissues for research | National regulations on ethical procurement and project approval |
| Human cells and tissues for transplantation and therapy | National and international regulations on ethical procurement, processing and storage ***(17)*** |
| Organisms and cell lines for manufacture of products for human therapy | International regulations for cell substrates ***(18,19)***<br>International regulations on proliferation of biological warfare |
| Organisms and cell lines for testing purposes | International regulations and protocols for product testing (e.g., OECD Good Laboratory Practice ***(20)***, Pharmacopeia monographs (http://www.usp.org/ or http://www.pheur.org/) |

storage temperatures. Many of the authors in this book have commented on long-term storage and a review of the literature gives many examples of successful storage of cryopreserved organisms for periods of up to many decades **(Table 3)**.

However, statements regarding sustained viability of cryopreserved cells is often based on the assumption that their storage temperature will be reliably maintained at that of liquid nitrogen (–196°C). Clearly, material stored in the vapor phase will never achieve this temperature and the actual storage temperature may well fluctuate because of variation in the levels of liquid nitrogen in storage vessels over time. Critically the storage temperature should not rise above the melting point of any cryopreserved cell suspensions or above the glass transition temperature of any vitrified material (*see* Chapters 2 and 3). Individuals searching for stored material may also move preserved preparations into ambient temperatures for brief periods, which if repeated over time could lead to loss of viability. In order to assure the long-term viability of stored cells and tissues it is therefore important to ensure that storage areas are well controlled with regular checks on liquid nitrogen levels and vessel-filling rotas, as well as appropriate staff training ***(18)***.

### *4.2. Storage Facilities*

Having invested time and resources in the quality of banks of cell cultures it is wise to provide a secure, clean, and stable environment for long-term storage that is also safe for laboratory staff to use ***(15)***. Security for stored material is assured through adoption of appropriate management systems to restrict access to authorized personnel, appropriate alarms for nitrogen storage vessels, and documented procedures for filling and maintenance of nitrogen storage. Monitoring in the form of temperature alarm systems and auditing to ensure correct maintenance and documentation are also important activities for BRC operation.

It is important to establish whether storage will be in the liquid or vapor phase of nitrogen or if electrical freezers (–100°C or below) are to be considered. In theory, the liquid phase of nitrogen provides the lowest and most stable storage temperature and is the method of choice for long-term storage. However, the risks of transmission of pathogenic virus should be considered as highlighted in past cases of patient deaths from bone marrow contaminated in storage ***(35)***. Vapor-phase storage may increase the risk of temperature cycling in stored materials but is generally more convenient and safer for regular access to stored material than liquid-phase storage. Some manufacturers (e.g., CBS) supply vapor-phase storage systems where liquid nitrogen is retained in the vessel walls, thus improving safety for laboratory staff. Electrical storage systems provide a very practical and maintenance-free low temperature storage solution. However, materials stored in such systems in a

**Table 3**
**Examples of Long-Term Viability of Preserved Organisms**

| Taxa/material | Stored | Storage duration | Comment | Reference |
|---|---|---|---|---|
| Bacteria | LN | 5–35 yr | Survival | ***22*** |
| 19 yeast strains | LN | long term | No reduction in viability | ***23*** |
| Yeast | FD | up to 30 yr | Survival | Chapter 6 |
| Fungi | FD | >30 yr | Good survival | ***24*** |
| *Schizo-saccharomyces pombe* | LN | 10 yr | Unchanged genetically | Chapter 7 |
| Various microalgae | LN | >20 yr | No reduction in viability | ***25*** |
| Various cyanobacteria | LN | >20 yr | No obvious loss of viability | Rippka (personal communication) |
| Lettuce seeds | LN | >10 yr | No loss viability half-life c. 3400 yr | ***26*** |
| Stem cells | LN | 15 yr | High efficiency of recovery | ***27*** |
| Human and sheep red cells | LN | 12 yr | No deterioration in function | Chapter 20 |
| Human hematopoietic stem cells | LN | 14 yr | Retain engraftment potential | ***28*** |
| Bovine sperm | LN | 37 yr | Normal motility and successful fertilization | ***29*** |
| Human sperm | LN | 5 yr | No statistical reduction in quality | ***30*** |
| Human sperm | LN | 21 yr | Successful fertilization and live birth | ***31*** |
| Canine islets of langerham | LN | 6 mo | No reduction in insulin secretion | ***32*** |
| Sheep embryos | LN | 5 yr | No reduction in live births vs 2 wk storage | ***33*** |
| Sheep embryos | LN | 13 yr | No reduction in live births vs 1 mo storage | ***34*** |

FD, freeze dried; LN, liquid nitrogen storage.

multiuser environment may suffer from regular disturbance to access material with the risk that critical materials may be lifted into ambient temperatures on a regular basis. Electrical freezers are also at risk where power supplies may not be reliable and even if power is reliable manufacturers often recommend liquid nitrogen or carbon dioxide back-up systems to cope with emergencies.

Appropriate facilities, equipment, and training in handling liquid nitrogen are vitally important for staff safety in cryostorage environments. In addition to the risks of frostbite from contact with ultra-low temperature equipment and liquids, staff should also be made aware of the risks of ampoule explosion (from trapped nitrogen liquid) and asphyxiation resulting from displacement of oxygen. The latter hazard is particularly important in enclosed storage areas and staff may need to be issued with personal oxygen monitors. Hazardous materials and those in quarantine should be stored separately, and it is also useful to separate material intended for archive storage and other material that will be accessed regularly.

### *4.3. Documentation of Stored Materials*

Accurate records of stored materials are vital to enable retrieval of ampoules of cells efficiently. They may also be a legal requirement where genetically modified, infectious, or other hazardous materials are stored. Numerous commercial database systems are available specially designed for this purpose, but it is important to select a system which is flexible to the full range of user requirements. It is wise to have up-to-date hard copy printouts or back-up electronic copies of these, and to ensure that amendments to storage records for additions or withdrawals can be made at the storage site to avoid transcriptional errors.

### *4.4. Other Issues for Long-Term Storage*

Over time liquid nitrogen freezers become clogged with a build up of ice sludge, which can accumulate microbial contamination from environmental sources ***(36)***. Thus, long-term storage vessels will benefit from periodic cleaning to remove the ice sludge and it is also helpful to carefully disinfect recovered ampoules. The use of double-sealing methods for ampoules or storage boxes will also help to provide protection against microbial contamination. Serious and lethal viral cross-infection of cells for transplantation from damaged containers in liquid nitrogen has also been reported ***(35)***, which emphasizes the importance of such procedures for all stored biological materials.

Natural radiation has also been considered a potential cause of loss of viability or mutation in stored cells and tissues. However, there does not appear to be any evidence for the adverse effects of long-term storage in well-maintained nitrogen vessels even for biological systems that might be expected to be more sensitive to such effects, such as embryos ***(37)***.

**Table 4**
**Typical Storage and Transport Conditions for Different Kinds of Cryopreserved Materials**

| Material | Storage conditions | Shipping conditions |
|---|---|---|
| Freeze-dried proteins, bacteria, and fungi | Generally stored at or below 4°C depending on thermal stability | Can be stored at ambient temperature depending on stability |
| Cryopreserved cells and organisms in vials or ampoules | Generally stored at temperatures below –100°C in electric freezers (–100 to –150°C) or in the liquid or vapor phase of liquid nitrogen (–160 to 196°C). Storage is possible at –80°C but viability will decrease with time ***(41)*** | Shipment can be made in dry-ice packages (–80°C). It is vital to ensure sufficient dry ice is included to keep the material frozen until receipt. Good shipping companies will ensure that packages are topped up with dry ice |
| Cryopreserved tissues and cells for transplantation | Generally stored in the vapor phase (not submerged in liquid) of liquid nitrogen to avoid contamination | Special shipment containers ("dry shippers") are often used to maintain temperature of vapor-phase LN |
| Cells preserved by vitrification (*see* Chapters 3 and 12) | Stored below the glass transition temperature (*see* Chapters 2 and 3) for the particular preservation method and cells | Shipment must also be below the glass transition temperature (e.g., in dry shippers containing adsorbed nitrogen liquid). Higher temperatures, such as dry ice, will cause devitrification and loss of viability |

LN, liquid nitrogen storage.

For important archive material it is wise to split such material between separate storage vessels and ideally to have an additional off-site storage location. It is clearly vital to monitor the quality of the storage environment for long-term sustained viability of stored cultures. Where much of the stored materials is of one type in terms of its potential stability in cryostorage, it may be helpful establish "sentinel" banks of representative cells stored in locations prone to temperature cycling (e.g., the upper regions of storage inventory systems) that are recovered periodically to detect any trends in viability levels ***(38)***. However, alternative physical temperature monitoring methods for key locations will generally be adequate, and RFID technology ***(39)*** is now delivering devices that could record and report the temperature experienced by individual vials or ampoules of cells in storage over time. In some circumstances accelerated thermal stability studies are used to predict the survival of stored biological materials at ultra-low temperature (*see* Chapters 4 and 13 ***[40]***). A variety of storage conditions can be obtained for biological materials and **Table 4** shows a simplified summary of suitable storage and transport conditions for different kinds of preserved biological materials.

## 5. Conclusion

The availability of quality controlled and authentic biological materials and cultures through professional BRCs is a significant advantage for science promoting standardization, efficiency, and laboratory safety. Culture strains become established in professional standards for industrial and biomedical work and may need to be stored stably for decades to support conservation programs, production processes, testing methods, and patents. BRCs may be challenged to provide cryostorage for large numbers of strains of organisms even when cryopreservation methods are not optimized for all organisms to be preserved. BRCs will continue to provide sources of specialist advice and training in the skills of preservation and culture that are increasingly needed with the development and expansion of cell-based in vitro experimentation.

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